

Preliminary Amendment  
Applicants: Boles *et al.*  
U.S.S.N.: Not Yet Assigned  
Filed: Herewith  
Atty. Docket No.: EXT-072C2  
Page 2

**Amendments to the Specification:**

On page 1 of the application, please amend the line beginning “Attorney Docket No.” as follows:

Attorney Docket No. ~~018422-000210~~ EXT-072C2 .

On page 1 of the application, please replace the title with the following title:

**~~DETECTION OF NON-VIRAL ORGANISMS WITH SRP RNA~~ UNIVERSAL GEL AND METHODS FOR USE THEREOF .**

Please replace the first full paragraph beginning on page 1, line 11, under the heading “CROSS-REFERENCE TO RELATED APPLICATIONS” with the following amended paragraph:

The present application is a continuation of U.S. Patent Application Serial No. 10/024,944, filed December 19, 2001, which is a continuation of U.S. Patent Application Serial No. 09/336,609, filed June 18, 1999, now abandoned, which claims priority to U.S. Patent Application Serial No. 60/090,063, filed June 19, 1998, and is related to U.S. Patent Application Serial No. 08/971,845, filed August 8, 1997, the entire disclosure of each of which is incorporated herein ~~both incorporated~~ by reference.

Please insert the following paragraph on page 14, between lines 12 and 13 of that page and after the first full paragraph beginning “In another embodiment”:

In some embodiments, nucleic acid probes for detecting bacteria include the following sequences: GCTGCTTCCGTC (SEQ ID NO:21); CGGACCTGACCTG (SEQ ID NO:22); AGGACCUGACAUG (SEQ ID NO: 23); CGGACCUGACCAG (SEQ ID NO: 24); CGGACCUGACAAG (SEQ ID NO:25); and CGGAUCUGACACG (SEQ ID NO: 26).

Please replace the paragraph beginning on page 18, line 4 with the following amended paragraph:

Enzymatic methods typically allow consistent release of nucleic acid from samples of small quantity, where physical contact for disruption cannot be assured. In [[a]] one embodiment, lyticase treatment (Sigma, St. Louis, MO) is used to disrupt the cell wall. Snail gut enzyme is the prototype enzyme used for cell wall lysis, but the preparation can have some variability in activity from batch to batch (Kitamura, *et al.*, *Journal of General Applied Microbiology* 18:57-71 (1972); Kitamura, *et al.*, *Journal of General Applied Microbiology* 20:323-344 (1974)). [[â]] $\beta$ -1,3-glucanase enzymes hydrolyze glucose polymers at [[â]] $\beta$ -1,3-glucan linkages to release laminaryipentaose and result in spheroplasts, modified organisms with partial loss of the cell wall and increased osmotic sensitivity (Pringle, *et al.*, *Journal of Bacteriology*, [[140]]140:289-293 (1979)). [[â]] $\beta$ -1,3-glucanase products available for use include, but are not limited to, zymolase (ICN Biomedicals, Costa Mesa, CA) (Kitamura, *et al.*, *Archives of Biochemistry & Biophysics* 153:403-406 (1972)), which is purified from a submerged culture of *Arthrobacter luteus* in the fermentation of yeast, and lyticase (Sigma, St. Louis, MO) (Scott, *et al.*, *Journal of Bacteriology* 142:414-423 (1980)), which is a genetically engineered synthetic equivalent. Zymolase is an impure product; other enzymes found in the preparation include [[â]] $\beta$ -1,3-gluconase, protease, mannanase, amylase, xylanase, phosphatase, and trace DNase. Use of synthetic product avoids these impurities.

Please replace the paragraph beginning on page 24, line 21 with the following amended paragraph:

Following electrophoreses, the gel was stained with ethidium bromide and RNA bands were visualized by UV illumination. Under these conditions, good separation of small RNAs was achieved. The gel was electroblotted onto a Hybond filter (Amersham) using a CBS Scientific blotting device according to manufacturer's instructions. The filter was baked at 80°C

for 2 hours, then hybridized to an oligonucleotide probe complementary to 4.5S RNA (*see* probe sequence, below). The probe was labeled with  $^{32}\text{P}$  by polynucleotide kinase and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . This probe is a 41 mer with 21 nucleotides at the 5' end which are complementary to the conserved 22 nucleotides found in the central region of 4.5S. The filters were hybridized in Rapidhyb (Amersham) for 1 hour at 42°C and then washed twice in 5x SSC, 0.1% SDS for 5 minutes at room temperature, once in 0.5x SSC, 0.1% SDS at room temperature for 5 minutes, and finally twice in 0.5x SSC, 0.1% SDS for 10 minutes at 42°C. For detection of the labeled bands by autoradiography, the filters were wrapped in clear film (Saranwrap) and exposed to X-ray film at room temperature (*see* Figure 1).

Please replace the paragraph beginning on page 25, line 10 with the following amended paragraph:

Total RNA was isolated from a log phase culture of *E. coli* by standard methods. The RNA was treated with DNase 1, phenol/chloroform extracted, ethanol precipitated and resuspended in 1% SDS. Concentration was measured using a spectrophotometer by UV absorbance (A260). RNA samples were electrophoresed on a 5% polyacrylamide gel (29:1 acrylamide:bis) containing 8M urea and 0.5x TBE (tris-borate EDTA) running buffer. Samples containing increasing amounts of RNA were prepared (0.15  $\mu\text{g}$ , 0.3  $\mu\text{g}$  and 0.6  $\mu\text{g}$ ) in 1x denaturing buffer (2x buffer = 2x TBE, 13% ficoll w/v, 0.01% bromophenol blue, 0.05% xylene cyanol FF and 7M urea) in total volume of 6  $\mu\text{l}$ . Samples were heated to 80°C for 2 minutes before loading. The gel was run for 1 hour at 100V at room temperature.

Please replace the paragraph beginning on page 25, line 20 with the following amended paragraph:

Following electrophoresis, the gel was stained with ethidium bromide and RNA bands were visualized by UV illumination. Under these conditions, good separation of small RNAs was achieved. The gel was electroblotted onto a Hybond filter (Amersham) using a CBS

Scientific blotting device according to manufacturer's instructions. The filter was baked at 80°C for 2 hours and cut in half. One half of the filter was hybridized to an oligonucleotide probe complementary to 5S rRNA labeled with <sup>32</sup>P by polynucleotide kinase and [[[[α]]γ-<sup>32</sup>P]ATP. Similarly, the other half of the filter (containing the same samples) was hybridized to a probe for *E. coli* 4.5S SRP RNA (probe 2nf). The filters were hybridized in Rapidhyb (Amersham) for 1 hour at 42°C and then washed twice in 5x SSC, 0.1% SDS for 5 minutes at room temperature and 0.5x SSC, 0.1 SDS for 10 minutes at 42°C. The filters were wrapped in clear film (Saranwrap) and exposed to X-ray film at room temperature.

Please replace the paragraph beginning on page 26, line 8 with the following amended paragraph:

Sequences of oligonucleotides used:

4.5S probe (2nf): GGCACACGCGTCATCTGC (SEQ ID NO: [[8]]9)

5S probe (66nf): CCACACTACCATCGGCGCT (SEQ ID NO: [[9]]20)

Please replace the paragraph beginning on page 28, line 4 with the following amended paragraph:

Aliquots were thawed, 20% sodium dodecyl sulfate was added to a final concentration of 1.4% in a total volume of 15.6 μl, and tubes were heated at 130°C for 10 minutes. Tubes were removed to room temperature for several minutes, and hybridization mix was added to a final volume of 20 μl with the following final concentrations: 120 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, 22.5 mM Tris (pH 8), 22.5 mM boric acid, 0.5 mM aurin tricarboxylic acid, 8mM Na phosphate, and 50 nM of each of the alkaline phosphate-conjugated reporter probes, RP-1 (5'-alkaline phosphatase-GCUGCUUCCUUC (SEQ ID NO: [[4]]27); underlined bases represent 2'-O-methyl RNA nucleotides) and RP-2 (5'-alkaline phosphatase-GCUGCUUCCGUC (SEQ ID NO: 14). These mixtures were warmed to 55°C for 10 minutes, then removed to room temperature and 4 μl of loading buffer (50% glycerol, 0.2% xylene cyanole, 0.2% bromphenol

blue) added. Half of each mixture was loaded onto a 5% polyacrylamide gel (89 mM Tris (pH 8.5), 27 mM phosphate buffer), made with 10  $\mu$ M of each of the following five acrydite-modified, 2'-O-methyl RNA capture probes, polymerized into the gel in a fashion similar to that described in Example III.

CP-1 5'-acrydite-TTTTTT-CGGACCUGACCUG (SEQ ID NO:15)

CP-2 5'-acrydite-TTTTTT-AGGACCUGACAUG (SEQ ID NO:16)

CP-3 5'-acrydite-TTTTTT-CGGACCUGACCAG (SEQ ID NO:17)

CP-4 5'-acrydite-TTTTTT-CGGACCUGACAAG (SEQ ID NO:18)

CP-5 5'-acrydite-TTTTTT-CGGAUCUGACACG (SEQ ID NO:19)

The gel was run at 30°C at 20 volts/cm for 30 minutes, rinsed in diethanolamine buffer (2.4 M diethanolamine, 1 mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, pH 10) for 10 minutes, then AttoPhos<sup>TM</sup> chemifluorescent substrate (Boehringer-Mannheim) was added for 10 minutes. The reaction was stopped by the addition of 1M Na phosphate (pH 7.2) and the fluorescent signal was scanned on a Molecular Dynamics Fluorimager 595 (*see* Figure 7). All nine of the listed bacterial species were detected with this probe set.